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Investigating the interaction of sunset yellow aggregates and 6-fluoro-2-naphthoic acid: increasing probe molecule complexity

*Jonathan R. Katz and Iain J. Day**

School of Life Sciences, University of Sussex, Falmer, Brighton, BN1 9QJ, UK

* To whom correspondence should be addressed:

Telephone: +44 1273 876622

Fax: +44 1273 876687

Email: i.j.day@sussex.ac.uk

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Abstract

The interaction of small molecules with non-covalent assemblies is of wide interest. The use of a magnetically-active reporter nucleus allows information to be obtained in the presence of spectral overlap or in cases of high dynamic range. In this paper we explore the interaction of a larger probe molecule, 6-fluoro-2-naphthoic acid with assemblies of sunset yellow using ^{19}F chemical shifts and diffusion NMR methods. Comparing the observations with previous studies using fluorophenols, 6-fluoro-2-naphthoic acid prefers to associate as clusters at the ends of the sunset yellow stacks.

Introduction

Small molecules are often used as reporters or probes of aggregated or assembled structures in the solution phase, such as the use of the dye thioflavin T to monitor the assembly of amyloid fibrils.[1, 2] The interaction of small molecules with larger assemblies is also important in areas such as gas storage in metal-organic frameworks,[3] or drug intercalation into the grooves of DNA helices.[4] Understanding the nature of these interactions is therefore important. Recently, we have described the investigation of the interaction between three structural isomers of fluorophenol and sunset yellow using NMR spectroscopy.[5] Sunset yellow is an azo-dye known to form large assemblies comprising tens to hundreds of molecules when in isotropic solution,[6, 7] and lyotropic liquid crystals at elevated concentrations.[8-10] Fluorophenol was chosen as a simple "probe" molecule with which to investigate the interaction of a small molecule with an aggregated species as it shares some structural similarity with sunset yellow, i.e. an aromatic phenyl group, a polar hydroxyl group, etc. It also contains a unique NMR-active reporter nucleus, in this case a single fluorine-19 atom.[5] The probe was added at a constant relative concentration of 1 mol% in order to avoid any potential disruptive influence on the underlying sunset yellow aggregation.[5] This study revealed a weak association between fluorophenol and sunset yellow. A tentative change-over in the mode of interaction was assigned, that is, from binding of the fluorophenol at the end of the sunset yellow stacks at low concentration, to its insertion into the stacks as a function of increasing sunset yellow concentration.[5]

In this paper we expand on our previous work by increasing the complexity of the probe molecule, whilst maintaining some structural congruency with sunset yellow,

shown in Figure 1. This naturally suggests the use of a probe molecule based on a naphthalene core, with 6-fluoro-2-naphthoic acid (FNA) being an ideal choice. This (larger) probe molecule also contains an ionisable group, mimicking the solubilising sulfonate groups of sunset yellow. The main focus of this work was to assess what influence a larger sized probe molecule had on its interaction with the sunset yellow aggregates. To achieve this complementary NMR approaches were employed and the results compared to those obtained previously using fluorophenol as the probe molecule.[5]

Materials and Methods

Materials

All chemicals were purchased from Sigma Aldrich (Dorset, UK), except deuterium oxide, which was obtained from either Sigma Aldrich or Goss Scientific Instruments (Cheshire, UK). Sunset yellow was purified by ethanol precipitation.[7, 10] A 74 mM stock solution of 6-fluoro-2-naphthoic acid in D_2O was prepared, to which 1.5 equivalents of NaOD were added to produce the corresponding sodium salt, improving aqueous solubility. 347 μL of this solution was added to 2.653 mL of a 961 mM sunset yellow solution in D_2O (concentration confirmed using UV/vis spectroscopy), yielding a final stock of 850 mM sunset yellow, containing 1 mol% FNA. Aliquots of this stock solution were taken and diluted to produce solutions of the required concentrations. The absence of any mesophase in the samples was confirmed by observation of a singlet in the ^2H NMR spectrum.[8]

NMR Spectroscopy

NMR data were acquired on a Varian VNMRS 600 spectrometer (Agilent Technologies Ltd., Yarnton, UK) using a X{^{1}H-^{19}F} broadband probe equipped with a *z*-gradient coil. ^{1}H spectra were acquired with 16k complex points spanning a spectral width of 9615.4 Hz, while ^{19}F spectra were obtained using 32k complex points over 28409.1 Hz. Diffusion NMR measurements were performed using the Oneshot sequence,[11] using the same spectral windows as the 1D spectra, with a diffusion labelling period of 100 ms, 16 or 32 gradient points (1.5 or 3 ms in length), equally spaced in g^2, between 0.0452 and 0.5650 T m^{-1} and data fitted to the appropriately modified Stejskal-Tanner equation.[11, 12] Data were processed using either Mestrenova (Santiago de Compostella, Spain) or DOSY Toolbox[12] as appropriate.

Data Analysis

The concentration dependent changes in chemical shift were modelled using the isodesmic model, as reviewed by Martin,[13] or a suitable modification of the isodesmic model including the incorporation of a second species present at low concentration.[5, 13] Diffusion data were processed with DOSY Toolbox[12] prior to analysis using the methods reported previously.[5] All data analysis was performed using the open source SciPy library of the python programming language.[14]

Results and Discussion

Chemical Shift Changes

The use of a magnetically-unique reporter nucleus, such as fluorine-19, on the probe molecule allows straightforward access to chemical shift changes upon its interaction with the aggregates, without the complicating presence of signals arising from the

aggregates themselves.[5] This is beneficial in cases where there is significant spectral overlap due to structural similarity, or dynamic range issues when the probe is at a much lower concentration than the aggregating species. The lattermakes identification of probe signals against a background of aggregate signals challenging. FNA was added at 1 mol% to a series of sunset yellow samples with varying concentrations within its isotropic phase. The resulting ^{19}F spectra are shown in Figure 2(a). The observation of a single resonance indicates that, if there is an interaction between the FNA and sunset yellow, it is in the fast exchange regime. An observed chemical shift would therefore be the population-weighted average of that in the free and bound species.[15] This is inline with previously reported results for the interaction of fluorophenol and sunset yellow.[5] The 3-bond ^{19}F-^{1}H coupling is observable in some of the spectra, being around 8 Hz in magnitude which is typical for $^{3}J_{FH}$ in a fluorinated aromatic compound.[16] This coupling becomes less well resolved at higher sunset yellow concentrations, due to more efficient transverse relaxation leading to an increase in the observed line width. This is a result of the increased sample viscosity caused by the presence of larger aggregates.[6]

The ^{19}F chemical shifts of FNA are plotted as a function of sunset yellow concentration in Figure 2(b). There is a noticeable change towards more negative chemical shifts with greater sunset yellow concentrations, indicating an increase in shielding. This is consistent with an interaction between FNA and the sunset yellow aggregates via π-π stacking interactions.[5, 17] This trend appears to reach a plateau towards higher concentrations, at approximately 550 mM. With increasing concentration from this point there is a very slight upturn in the observed ^{19}F chemical shift. These ^{19}F chemical shift data can be interpreted using a modified isodesmic

model, to account for incorporation of a second species into the assembly of another, more prevalent species.[13] This model comprises two binding modes hence two equilibrium constants: one for the interaction of FNA with the ends of the sunset yellow stacks K_1, and a second for its incorporation into the assemblies K_2.[5, 13] The value of equilibrium constant (K_{eq} = 6.7 M^{-1}) for the associate of sunset yellow in the absence of the FNA was used as determined previously.[5] The results of this analysis are plotted as a solid line on Figure 2(b), with the equilibrium constants obtained given in Table 1. The corresponding data using 3FP as the probe molecule is included for comparison.[5] These data would suggest that the interaction of FNA with the sunset yellow assemblies is predominantly with the ends of the stacks. This type of interaction persists across a wide range of concentrations, before insertion into the stacks starts to occur at higher SSY concentrations (above 550 mM). These chemical shift changes, arising from the interaction of FNA with sunset yellow, are in contrast to those observed in the previous study of the various isomers of fluorophenol.[5] In the latter case, the initial decrease in chemical shift occurred up to sunset yellow concentrations of around 100 mM where upon the switch to increasing chemical shift, i.e. decreased shielding, occurred.[5] This behaviour was interpreted as a change in binding mode of the fluorophenol, from mainly interacting with the ends of the sunset yellow stacks at low concentrations, to being incorporated into the interior of the assemblies above a critical concentration.[5] A potential explanation for the different behaviour observed for FNA is that, unlike fluorophenol, FNA shows evidence of self-aggregation at the concentrations used in this study. Figure 2(c) plots the ^{19}F chemical shifts for the FNA samples in the absence of sunset yellow. There is a decrease in the observed chemical shift of around 0.13 ppm over this extended concentration range, indicative of (weak) self-aggregation in planar aromatic

molecules driven via π-π stacking interactions.[13, 17] Using the isodesmic model,[13] these data yield an equilibrium constant of 0.76 ± 0.73 M^{-1} for the self-association, which is consistent with that reported for the self association of other small aromatic molecules.[18, 19] While both 1- and 2-naphthoic acids are known to form stacked structures in the solid state,[20] the solution phase behaviour, and the role of the fluorine substituent, are the subject of on-going investigations.

Diffusion Measurements

The use of diffusion NMR methods to gain information on molecular size in solution, and by inference the association state, is well documented, with examples ranging from protein aggregation[21, 22] to the oligomeric state of organometallic complexes.[23] The investigation of sunset yellow aggregation by diffusion NMR[6] has provided complementary information to other physical techniques such as optical[7] or X-ray scattering.[7, 10, 24] Using changes in the diffusion properties of either the aggregates or a small probe molecule information on the nature of any interaction between the two can be obtained. Figure 3(a) shows the result of a series of diffusion NMR measurements on samples of sunset yellow with 1 mol% FNA, plus various control samples as discussed below. The addition of the FNA probe causes a small, but consistent, increase in the observed diffusion coefficient for sunset yellow, compared to the data in the absence of FNA, as seen by the filled circles and open triangles respectively. The change is of the order of 0.2×10^{-10} m^2 s^{-1}, and is more pronounced at lower concentrations. This change is to larger diffusion coefficients indicates the presence of smaller assemblies in the solution, suggesting that there maybe a slight destabilisation of the sunset yellow aggregates on addition of the FNA probe, even at the low relative concentration of 1 mol%. Using the ^{19}F nucleus allows access to the

FNA diffusion properties in a background-free manner. These data give diffusion coefficients, plotted as the open circles in Figure 3(a), which are larger than those measured for sunset yellow, indicating that there is not complete association between the probe and the aggregates. This behaviour closely follows that observed previously for the interaction of the fluorophenol probes.[5] In the absence of sunset yellow, the FNA diffusion coefficients do show a very slight decreasing trend with increasing concentration, consistent with the self-assembly observed above from the ^{19}F chemical shifts. In order to account for the effect of increasing sample viscosity caused by the presence of the sunset yellow aggregates a viscosity correction can be applied,[5] i.e. observed diffusion coefficients of the FNA in the absence of sunset yellow are scaled by the ratio of the solvent diffusion coefficient in the presence, D_{HOD}, and absence, D_{HOD}^{0}, of sunset yellow:

$$D_{\mathrm{FNA,free}}^{\mathrm{corr}} = \frac{D_{\mathrm{HOD}}}{D_{\mathrm{HOD}}^{0}} D_{\mathrm{FNA,free}} \qquad (1)$$

This procedure gives rise to the open squares plotted in Figure 3(a). Clearly, these points do not directly overlay the observed ^{19}F diffusion coefficients for FNA outlined previously (open circles). The diffusion properties measured for the FNA in the presence of sunset yellow are therefore not solely the result of increased microscopic viscosity, but arise from interaction between FNA and the sunset yellow aggregates as seen previously for the isomers of fluorophenol and sunset yellow.[5]

The association of FNA with the sunset yellow assemblies occurs in the fast exchange regime on the time scale of the diffusion labelling period, therefore, the observed diffusion coefficient for the FNA probe is the populate-weighted average of the diffusion coefficients for the aggregates and the free FNA in solution.[5, 25] Using this information allows for the mole fraction of bound probe molecules χ_{asc} to be

calculated using the approach described previously.[5] The results of this analysis are shown in Figure 3(b) and reveals a similar biexponential-shaped profile to that observed when investigating the interaction of fluorophenol with sunset yellow. The parameters for a biexponential fit of the mole fraction as a function of concentration are given in Table 2. The relative amplitudes of the two components 47% and 53% for the a_1 and a_2 respectively, is similar to those for 3-fluorophenol[5] which is also shown in Figure 3(b) for comparison. There are two distinct differences between the FNA and 3FP data, the first is the point at which the change over in exponential component occurs, around 70 mM for 3FP,[5] but closer to 120 mM in the case of FNA. The second is the much larger fraction of FNA bound to the sunset yellow aggregates across all concentrations compared to fluorophenol. For example for the 400 mM sunset yellow sample, approximately 70% of the FNA molecules are bound to the aggregates, compared with only ~45% for the various structural isomers of fluorophenol.[5] It is plausible that the increased π-π overlap caused by the larger size of the FNA molecules is responsible for the greater overall binding of the probe molecules.

Taken together, the results of the chemical shift and diffusion measurements performed here can be interpreted as follows. The chemical shift changes indicate that the binding of the FNA molecules is principally to the ends of the sunset yellow stacks, while the diffusion measurements suggest that a large fraction of the FNA molecules in solution are bound to the sunset yellow aggregates. This bound fraction is generally much larger than that number of available stack ends at all but low sunset yellow concentrations, i.e. above 35 mM. This is demonstrated in Figure 4, using the

association model of Israelachvili[26] previously used to interpret sunset yellow aggregation.[6] The fraction of stack ends available can be written as:

$$f_{\text{end}} = \sum \frac{X_N}{N} \frac{2}{N} \tag{2}$$

where X_N/N is the number of aggregates comprising N molecules and $2/N$ is the proportion of ends available. This number fraction is determined from NMR diffusion data of sunset yellow as reported.[6] The fraction of associated FNA molecules being larger than the number of available stack ends, combined with the fact that FNA appears to show evidence of self-aggregation, indicates that clusters or assemblies of FNA molecules are likely to bind to the ends of the sunset yellow stacks at concentrations below around 550 mM. It is only at much higher concentrations, that is in the presence of much larger sunset yellow assemblies, that incorporation of FNA into the sunset yellow stacks begins to occur.

Conclusions

Sunset yellow presents an ideal system with which to investigate small molecule aggregation as a function of sample composition.[6, 7, 10, 27] Previous work has investigated the interaction of a series of structurally isomeric fluorophenols with sunset yellow.[5] This revealed that the addition of the probe species resulted in little disruption to the aggregates, and postulated changes in binding mode as a function of concentration. This work extends this idea to the use of a larger, but still structurally similar probe, 6-fluoro-2-naphthoic acid. While the general trends are similar between the two probe species, FNA does show some distinct differences. Whilst a greater proportion of FNA appears to be bound to the sunset yellow aggregates, the major binding mode is to the ends of the stacks. This end-on binding is likely via clusters of FNA molecules, given the evidence of self-association that was not present in the case

of the fluorophenols. The influence of a fluorine substituent on π-π driven aromatic stacking is currently under investigation.

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Tables

Table 1: Equilibrium constants obtained from fitting a modified isodesmic model[13] to the data presented in Figure 2. The corresponding data for 3-fluorophenol is included for comparison.[5]

Sample	K_1 (M^{-1})	K_2 (M^{-1})
FNA	0.03 ± 0.18	0.20 ± 9.75
3FP[5]	0.1 ± 0.1	1.3 ± 3.8

Table 2: Parameters from a biexponential fit of the function $\chi_{asc} = a_1 \exp(-c_T/b_1) + a_2 \exp(-c_T/b_2) + a_0$ to the data presented in Figure 3(b). The corresponding data for 3-fluorophenol is included for comparison.[5]

Sample	a_1	b_1 (M)	a_2	b_2 (M)	a_0
FNA	-0.442	0.058	-0.603	0.754	1.04
3FP[5]	-0.639	0.017	-0.917	1.096	1.09

Figure Captions

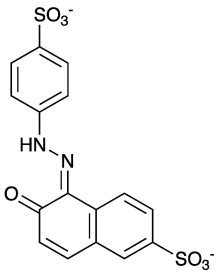
Figure 1: Structure of the major azo tautomer of sunset yellow **1** and 6-fluoro-2-naphthoic acid **2**.

Figure 2: (a) ^{19}F NMR spectra of 1 mol% 6-fluoro-2-naphthoic acid in solutions of various concentrations of sunset yellow. (b) ^{19}F Chemical shifts of FNA as a function of sunset yellow concentration in the presence (filled circles) of sunset yellow, (c) FNA in the absence (open circles) of sunset yellow. The solid lines are fits to an

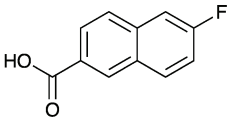
isodesmic model,[13] including the addition of a second species at low relative concentration in the case of the filled symbols.

Figure 3: (a) Diffusion coefficients for sunset yellow with and without 1 mol% FNA as a function of sunset yellow concentration. The viscosity correction is performed by scaling the FNA diffusion coefficient by the ratio of the solvent (HOD) diffusion coefficient in the present and absence of sunset yellow. (b) Associated fraction of probe molecules as a function of sunset yellow concentration. For comparison, the data for 3-fluorophenol is also plotted.[5]

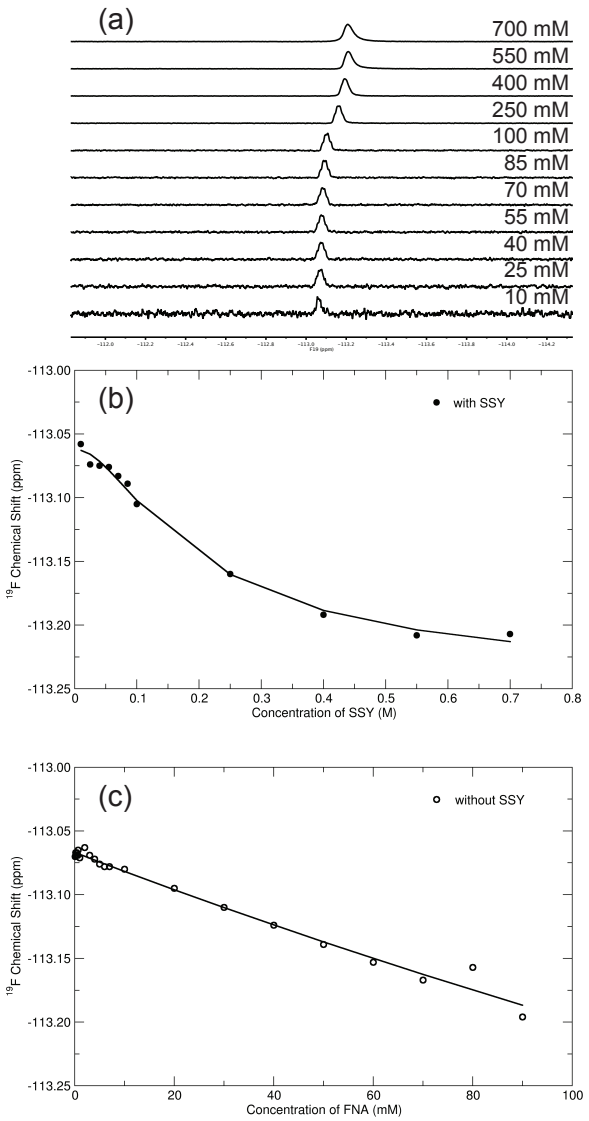
Figure 4: Fraction of FNA associated with the sunset yellow aggregates and the fraction of stack ends available, calculated using eq 2, as a function of sunset yellow concentration. The average number of molecules per aggregate was calculated using the data of Renshaw and Day.[6]

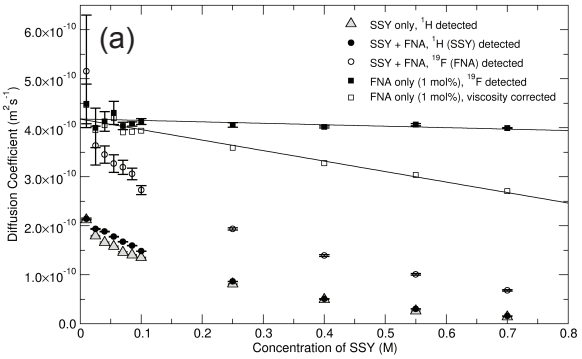


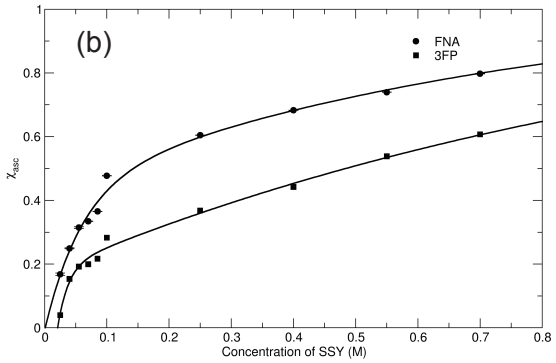
1

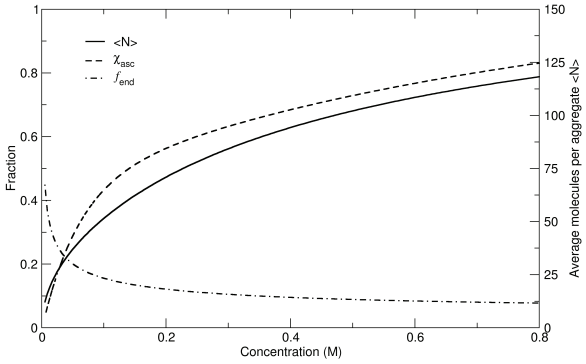


2


(a)
700 mM
550 mM
400 mM
250 mM
100 mM
85 mM
70 mM
55 mM
40 mM
25 mM
10 mM
(b)
with SSY
^{19}F Chemical Shift (ppm)
Concentration of SSY (M)
(c)
without SSY
^{19}F Chemical Shift (ppm)
Concentration of FNA (mM)


(a)
SSY only, ^{1}H detected
SSY + FNA, ^{1}H (SSY) detected
SSY + FNA, ^{19}F (FNA) detected
FNA only (1 mol%), ^{19}F detected
FNA only (1 mol%), viscosity corrected
Diffusion Coefficient ($m^2 s^{-1}$)
6.0×10^{-10}
5.0×10^{-10}
4.0×10^{-10}
3.0×10^{-10}
2.0×10^{-10}
1.0×10^{-10}
0.0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Concentration of SSY (M)


(b)
FNA
3FP
χ_{asc}
1
0.8
0.6
0.4
0.2
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Concentration of SSY (M)


<N>
χ_{asc}
f_{end}
Fraction
Average molecules per aggregate <N>
Concentration (M)
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0.2
0.4
0.6
0.8
1
0
25
50
75
100
125
150